

Exhibit A

Plants with increased activity of a Class 3 branching enzyme***Description***

[001] The present invention relates to plant cells and plants, which are genetically modified, wherein the genetic modification leads to an increase in the activity of a Class 3 vegetable branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified. Furthermore, the present invention relates to means and methods for the manufacture of such plant cells and plants. Plant cells and plants of this type synthesise a modified starch. The present invention therefore also relates to the starch synthesised by the plant cells and plants according to the invention as well as to methods for the manufacture of the starch and to the manufacture of starch derivatives of this modified starch.

[002] With regard to the increasing importance currently attributed to vegetable constituents as renewable raw material sources, one of the tasks of biotechnological research is to endeavour to adapt these vegetable raw materials to suit the requirements of the processing industry. Furthermore, in order to enable regenerating raw materials to be used in as many areas of application as possible, it is necessary to achieve a large variety of materials.

[003] Polysaccharide starch is made up of chemically uniform base components, the glucose molecules, but constitutes a complex mixture of different molecule forms, which exhibit differences with regard to the degree of polymerisation and branching, and therefore differ strongly from one another in their physical-chemical characteristics. Discrimination is made between amylose starch, an essentially unbranched polymer made from α -1,4-glycosidically linked glucose units, and the amylopectin starch, a branched polymer, in which the branches come about by the occurrence of additional α -1,6-glycosidic links. A further essential difference between amylose and amylopectin lies in the molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of $5 \times 10^5 - 10^6$ Da, that of the amylopectin lies between 10^7 and 10^8 Da. The two macromolecules can be differentiated by their molecular

weight and their different physical-chemical characteristics, which can most easily be made visible by their different iodine bonding characteristics.

[004] Amylose has long been looked upon as a linear polymer, consisting of α -1,4-glycosidically linked α -D-glucose monomers. In more recent studies, however, the presence of α -1,6-glycosidic branching points (ca. 0.1%) has been shown (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

[005] Amylopectin constitutes a complex mixture of differently branched glucose chains. In contrast to amylose, amylopectin is more strongly branched. According to textbook information (Voet and Voet, Biochemistry, John Wiley & Sons, 1990), on average, the α -1,6 branches occur every 24 to 30 glucose residues. This is equivalent to a degree of branching of ca. 3% - 4%. The figures for the degree of branching are variable and are dependent on the origin (e.g. plant species, plant type etc.) of the appropriate starch. In typical plants used for the industrial production of starch, such as maize, wheat or potato, for example, the synthesised starch consists of ca. 20% - 30% amylose starch and ca. 70% - 80% amylopectin starch.

[006] The functional characteristics of the starch, along with the amylose/amyopectin ratio and the phosphate content, are strongly affected by the molecular weight, the pattern of the side chain distribution, the ion concentration, the lipid and protein content, the average grain size of the starch and the grain morphology of the starch etc. At the same time, by way of example, the solubility, the retrogradation behaviour, the water bonding capability, the film formation characteristics, the viscosity, the sticking characteristics, the freezing-thawing stability, the acid stability, the gelling strength etc. must be mentioned as important functional characteristics. The grain size of the starch can also be important for different applications.

[007] Branching enzymes, which are also abbreviated by the designation "BE" (from Branching Enzyme; E.C. 2.4.1.18), catalyse the introduction of α -1,6 branches in α -1,4-glucans. Branching enzymes and the nucleic or amino acid sequences that characterise them are known from widely different organisms, such as bacteria, microbial fungi, mammals, algae and higher plants, for example. As only plants synthesise starch, while the above-mentioned non-vegetable

organisms (e.g. bacteria, fungi and mammals) synthesise glycogen, the related branching enzymes, which are involved in the synthesis of the appropriate polymer, can also be sub-divided into glycogen branching enzymes and starch branching enzymes. Plants are therefore starch branching enzymes, which are often also referred to as Q-enzymes in older literature.

[008] In all plant species that have been investigated up to now, the branching enzymes described can be associated with two different classes (Burton et al., 1995, Plant Journal 7, 3-15; Mizuno et al., 2001, Plant Cell Physiol. 42(4), 349-357). The association with these classes, sometimes designated in the literature with A or 2 respectively and B or 1 respectively, is based on the comparison of derived protein sequences.

[009] As different nomenclatures have been used in the past for designating and classifying branching enzymes, Smith-White and Preiss (1994, Plant Molecular Biology Reporter 12, 67-71) (1994, Plant Molecular Biology Reporter 12, 67-71) have proposed a system for standardising this nomenclature, in which the association with the two classes of vegetable branching enzymes is also based on the comparison of derived protein sequences (Larsson et al., 1998, Plant Mol. Biol. 37, 505-511). According to this nomenclature, those vegetable branching enzymes, the amino acid sequence of which has a higher degree of identity with that of branching enzyme I of maize (GenBank Acc: D11081), is to be designated as a Class 1 branching enzyme, and those vegetable branching enzymes, the coding amino acid sequence of which has a higher degree of identity with that of branching enzyme II of maize (GenBank Acc: AF072725), is to be designated as a Class 2 branching enzyme. The designation of gene products, which are coding for branching enzymes, are, in accordance with the nomenclature of Smith-White and Preiss, to be incorporated in the already existing nomenclature by means of E.C. numbers. This results in the so-called GPN (Gene Product Number) Codes for the two classes, namely GPN 2.2.4.1.18:1 for Class 1 branching enzymes and GPN 2.2.4.18:2 for Class 2 branching enzymes.

[0010] The following vegetable or starch branching enzymes therefore belong to Class 1 (GPN 2.2.1.18:1) according to the nomenclature proposed by Smith-White and Preiss (1994, Plant Molecular Biology Reporter 12, 67-71): BE I from *Aegilops tauschii* (GenBank Acc: AF525746), BE I from barley (GenBank Acc: AY304541), BE from tapioca (GenBank Acc:

X77012), BE I (frequently also described as BE 1) from rice (GenBank Acc: D11082, D10752, D10838), BE 3 from bean (GenBank Acc: AB029549), BE II from pea (GenBank Acc: X80010), BE from millet (GenBank Acc: AF169833), BE I from potato (GenBank Acc: Y08786, X69805), BE from wheat (GenBank Acc: Y12320, AF076679, AF002820) and BE I from maize (GenBank Acc: D11081, AAO20100, E03435, AY176762, U17897, AF072724). At the same time, the amino acid sequences for different Class 1 branching enzymes each have an identity of more than 60% with the amino acid sequence of branching enzyme I from maize (GenBank Acc: D11081).

[0011] Branching enzymes, which belong to Class 2 (GPN 2.2.1.18:2) according to the nomenclature proposed by Smith-White and Preiss (1994, Plant Molecular Biology Reporter 12, 67-71) are, for example, BE IIa from *Aegilops tauschii* (GenBank Acc: AF338431, WO 9914314), BE2-1 and BE2-2 from *Arabidopsis thaliana* (BE2-1 GenBank Acc: NM_129196 CAA04134; BE2-2 GenBank Acc: CAB82930, NM_120446), BE IIa and BE IIb from barley (BE IIa GenBank Acc: AF064560; BE IIb GenBank Acc: AF064561), BE II from sweet potato (GenBank Acc: AB071286), BE III and BE IV (frequently also described as BE 3 or BE 4 respectively) from rice (BE III GenBank Acc: D16201; BE IV GenBank Acc: AB023498), BE 1 from bean (GenBank Acc: AB029548), BE I from pea (GenBank Acc: X80009), BE IIb from millet (GenBank Acc: AY304540), BE II from potato (GenBank Acc: AJ000004, AJ011885, AJ011888, AJ011889, AJ011890), BE II or BE IIa from wheat (GenBank Acc: Y11282, AF286319, AF338432, U66376) and BE II, or BE IIb from maize (BE II GenBank Acc: AAA18571, T02981; BE IIb GenBank Acc: AF072725, L08065). At the same time, the amino acid sequences for different Class 2 branching enzymes each have an identity of more than 60% with the amino acid sequence of branching enzyme IIb from maize (GenBank Acc: AF072725).

[0012] Vegetable or starch branching enzymes belong to the family of alpha-amylolytic enzymes (Svensson, 1994, Plant Molecular Biology 25, 141-157; Jespersen et al., 1991, Biochem J. 280, 51-55) and, with regard to the amino acid sequence, have four conserved domains (Baba et al., 1991, Biochem. Biophys. Res. Commun. 181(1), 87-94; Kuriki et al., 1996, J. of Protein Chemistry 15(3), 305-313).

[0013] Structural predictions based on mathematical calculations derived from experimental

data such as protein crystal structures (Pfam: <http://hits.isb-sib.ch/cgi-bin/PFSCAN?>) show that all previously known branching enzymes from higher plants have two domains: an alpha-amylase domain and an iso-amylase domain. Here, the iso-amylase domain lies closer to the N-terminus of the protein than the alpha-amylase domain.

[0014] Plants are known, for example, which have a reduced activity of a Class 2 branching enzyme due to a mutation. These include the so-called "*amylose extender*" (*ae*) mutants from maize (Standard et al., 1993, Plant Cell 5, 1555-1566; Boyer and Preiss, 1978, Biochem. Biophys. Res. Commun. 80, 169-175) and rice (Mizuno et al., 1993, J. Biol. Chem. 268, 19084-19091), as well as the "*rugosus*" (*r*) mutation in pea (Smith, 1988, Planta 175, 270-279; Bhattacharyya et al., 1990, Cell 60, 115-122). All these mutants are distinguished by the fact that they synthesise a starch, which has an increased amylose content in comparison with starches from corresponding plants, which do not have this mutation.

[0015] Furthermore, genetically modified potato plants are described, in which the activity of a BE I (Class 1) branching enzyme (Kossmann et al., 1991, Mol Gen Genet 230, 39-44; Safford et al., 1998, Carbohydrate Polymers 35, 155-168), or the activity of a BEII (Class 2) branching enzyme (Jobling et al., 1999, The Plant Journal 18), or the activity of a BEI and BEII branching enzyme (Schwall et al., 2000, Nature Biotechnology 18, 551- 554, Jobling et al., 2003, Nature Biotechnology 21, 77-80) are reduced.

[0016] Previously, it has been possible to associate all vegetable branching enzymes to one or both of the classes described above. Plant cells or plants, which have an increased activity of a branching enzyme, which cannot be associated with these classes, are unknown.

[0017] The object of the present invention is therefore based on providing modified starches, new plant cells and/or plants, which synthesise such a modified starch, as well as methods for producing said plants.

[0018] This problem is solved by the embodiments described in the claims.

[0019] The present invention therefore relates to genetically modified plant cells and plants, characterised in that the plant cells or plants have an increased activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

[0020] A first aspect of the present invention relates to a plant cell or plant, which is genetically modified, wherein the genetic modification leads to an increase in the activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

[0021] At the same time, the genetic modification can be any genetic modification, which leads to an increase in the activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

[0022] In conjunction with the present invention, the term "wild type plant cell" means that the plant cells concerned were used as starting material for the manufacture of the plant cells according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant cell according to the invention.

[0023] In conjunction with the present invention, the term "wild type plant" means that the plants concerned were used as starting material for the manufacture of the plants according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant according to the invention.

[0024] In conjunction with the present invention, the term "corresponding" means that, in the comparison of several objects, the objects concerned that are compared with one another have been kept under the same conditions. In conjunction with the present invention, the term "corresponding" in conjunction with wild type plant cell or wild type plant means that the plant cells or plants, which are compared with one another, have been raised under the same cultivation conditions and that they have the same (cultivation) age.

[0025] The plant cells according to the invention have an increased activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells that have not been genetically modified.

[0026] Here, within the framework of the present invention, the term "increased activity" means an increase in the expression of endogenous genes, which code Class 3 branching enzymes and/or an increase in the quantity of Class 3 branching enzyme protein in the cells and/or an increase in the enzymatic activity of Class 3 branching enzymes in the cells.

[0027] The increase in the expression can, for example, be determined by measuring the quantity of transcripts coding Class 3 branching enzyme, e.g. using Northern blot analysis or RT-PCR. Here, an increase preferably means an increase in the amount of transcripts in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%.

[0028] The increase in the amount of protein of a Class 3 branching enzyme, which results in an increased activity of this protein in the plant cells concerned, can, for example, be determined by immunological methods such as Western blot analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio Immune Assay). Here, an increase preferably means an increase in the amount of Class 3 branching enzyme protein in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%.

[0029] Within the framework of the present invention, the term "branching enzyme" (α -1,4-glucan: α -1,4-glucan 6-glycosyltransferase, E.C. 2.4.1.18) is understood to mean a protein, which catalyses a transglycosylation reaction, in which α -1,4 links of an α -1,4-glucan donor are hydrolysed and the thereby released α -1,4-glucan chains are transferred to an α -1,4-glucan acceptor chain and, in doing so, are transformed into α -1,6-links. In particular, within the framework of the present invention, the term "branching enzyme" is to be understood to mean a vegetable branching enzyme, i.e. a starch branching enzyme.

[0030] The activity of a branching enzyme can be demonstrated, for example, with the help of native acrylamide gel electrophoresis. In doing so, proteins are first separated electrophoretically and, after incubation in buffers containing an activity, which synthesises linear α -1,4-glucan chains (e.g. starch phosphorylase a) and its substrate (e.g. glucose-6-phosphate), the corresponding gels are coloured with iodine (Kimihiro et al., 1980, Analytical Biochemistry 108, 16-24). Furthermore, branching enzymes in microbial organisms, such as the *E. coli* strain KV832 for example (Kiel et al., 1987 Mol. Gen. Genet 207: 294-301), which do not synthesise branched α -glucans, can be expressed. If an activity of a branching enzyme is introduced into the microbial organism due to the expression of a foreign gene in such strains (e.g. *E. coli* KV832), then the branching enzyme activity can be demonstrated by treating colonies of these organisms with iodine vapour, for example. Colonies, which synthesise linear α -1,4-glucans, turn blue in this test, while colonies, which synthesise branched glucans by expressing an additional enzymatic activity of a branching enzyme, turn reddish-brown after treating with iodine vapour. It is also possible to express proteins in phosphoglucomutase mutants of *E. coli* to identify a branching enzyme activity of appropriate proteins (Buettcher et al., 1999, Biochem. Biophys. Acta 1432, 406-412).

[0031] A further possibility of demonstrating branching enzyme activity of proteins is the use of a reaction stimulated by phosphorylase a and the subsequent separation of the products by means of thin film chromatography (Almstrupp et al., 2000, Analytical Biochemistry 286, 297-300). Branching enzyme activities can also be demonstrated with the help of the methods described in Guan and Preiss (1993, Plant Physiol. 102. 1269- 1273) and Kuriki et al. (1996, J. of Protein Chemistry 15, 305-313).

[0032] In conjunction with the present invention, the term "Class 3 branching enzyme" is to be understood as a branching enzyme, which has a higher degree of identity with the amino acid sequence specified in Seq ID NO 4 than with that of the branching enzyme BE I from maize (GenBank Acc: D11081) or with that of the branching enzyme BE IIb from maize (GenBank Acc: AF072725). Preferably, the Class 3 branching enzyme comes from starch-storing plants, particularly preferably from plant species of the genus *Solanum*, especially preferably from *Solanum tuberosum*.

[0033] In a further embodiment of the present invention, amino acid sequences coding Class 3 branching enzymes have an identity of at least 60% with the sequence specified in SEQ ID NO 4, in particular of at least 70%, preferably of at least 80% and particularly preferably of at least 90% and especially preferably of at least 95%.

[0034] According to the invention, Class 3 branching enzymes have an iso-amylase domain (Pfam acc.: Pf02922) and an alpha-amylase domain (Pfam acc: Pf00128). According to the invention, the iso-amylase domain and the alpha-amylase domain in amino acid sequences coding branching enzymes are separated from one another by the presence of further amino acids, which do not belong to these two domains.

[0035] Class 3 branching enzymes according to the invention are distinguished by the fact that the iso-amylase domain is separated from the alpha-amylase domain by a greater number of amino acids than the iso-amylase domain and the alpha-amylase domain of Class 1 and 2 branching enzymes.

[0036] Class 3 branching enzymes according to the invention are preferably distinguished with regard to their amino acid sequence by the fact that they have at least 70, preferably at least 100, particularly preferably at least 130 and especially preferably at least 198 amino acids between the iso-amylase domain and the alpha-amylase domain. In a further embodiment of the present invention, in the case of an amino acid sequence coding a Class 3 branching enzyme, the C-terminal end of the iso-amylase domain is separated from the N-terminal beginning of the alpha-amylase domain by 70 to 198, preferably by 100 to 198, particularly preferably by 130 to 198 and especially particularly preferably by 150 to 198 amino acids.

[0037] With the help of the Pfam database (Batemann et al., 2002, Nucleic Acids Research 30, 276-280; accessible via <http://www.sanger.ac.uk/Software/Pfam/>, <http://www.cgb.ki.se/Pfam/>; <http://pfam.jouy.inra.fr/> or <http://pfam.wustl.edu/>), it is possible for the person skilled in the art to determine whether amino acid sequences already have known domains (e.g. an iso-amylase domain and/or an alpha-amylase domain). Pfam is a database put

together by experts, which classifies amino acid sequences into so-called families. Here, the assignment of an amino acid sequence to a family is carried out on the basis of so-called domains, which are to be looked upon as functional and structural components of proteins. A domain is defined as a structural unit or a repeatedly occurring amino acid sequence unit, which can occur in proteins with widely different functions. Along with information relating to the amino acid sequence of known proteins, further knowledge (e.g. evidence of the enzymatic activity, crystal structure data) is also used for the assignment of a protein to a family. Each family is assigned a name and an "accession" number (e.g. Name: Isoamylase_N, acc: PF02922). A constituent part of each family in the Pfam database is, amongst other things, a so-called "seed alignment". The "seed alignment" contains the amino acid sequences of representative proteins of a family. Starting from "seed alignments", a so-called profile HMM ("profile Hidden Markov Model"; overview article in: Durbin et al., "Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids", Cambridge University Press, 1998, ISBN 0-521-62041-4) is produced using the HMMER 2 software (freely available under <http://hmmer.wustl.edu/>). The HMMs produced have names and are stored in the Pfam database specifically for the correspondingly assigned domains. In contrast to classical, multiple "alignments" (e.g. produced using the Clustal W program or the Blosum62 algorithm), HMMs are based on a valid statistical theory (Bayes theory of conditional probability, Markoff chains) and enable an interrogation sequence (query) to be assigned to a family based on the use of position-specific evaluation matrices. This enables an assignment to be made even when there are considerable differences in the amino acid sequences between the interrogation sequence (Query) and a comparison sequence (e.g. amino acid sequence entry in a database).

[0038] The domain structure of the amino acid sequence concerned can be determined by means of a comparison of the HMMs stored in the Pfam database with amino acid sequences, which are entered as a so-called interrogation sequence (query) (e.g. under <http://hits.isb-sib.ch/cgi-bin/PFSCAN?>).

[0039] In conjunction with the present invention, the term "iso-amylase domain" is to be understood to mean a Pfam iso-amylase domain (acc: Pf02922). At the same time, the HMM describing this Pfam iso-amylase domain is to be produced with the HMMER 2 [2.3.1] software,

starting from a "seed alignment", which contains the amino acid sequences shown in Table 1. In conjunction with the present invention, the "seed alignment" is produced by means of the ClustalW program (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680; see below). The following settings must be chosen to produce the appropriate HMMs: Build Method of HMM: hmmbuild -F HMM_ls, hmmcalibrate -seed 0 HMM_ls; Gathering cutoff: 2.3 2.3; Trusted cutoff: 2.3 2.2; Noise cutoff: 2.1 2.1). Further information for producing the HMM of the Pfam iso-amylase domain (acc: Pf02922) is given in Table 3.

[0040] In conjunction with the present invention, the term "alpha-amylase domain" is to be understood to mean a Pfam alpha-amylase domain (acc: Pf00128). At the same time, the HMM describing this Pfam alpha-amylase domain is to be produced with the HMMER 2 [2.3.1] software, starting from a "seed alignment", which contains the amino acid sequences shown in Table 2. Here, the "seed alignment" is produced by means of HMM_simulated_annealing ([http://www.psc.edu/general/software/packages/hmmer/manual/node11.html#SECTION00321000000000000000](http://www.psc.edu/general/software/packages/hmmer/manual/node11.html#SECTION003210000000000000)). The following settings must be chosen to produce the appropriate HMM: Build Method of HMM: hmmbuild -F HMM_ls, hmmcalibrate -seed 0 HMM_ls; Gathering cutoff: -82.0 -82.0; Trusted cutoff: -81.7 -81.7; Noise cutoff: -82.7 -82.7). Further information for producing the HMM of the Pfam alpha-amylase domain (acc: Pf00128) is given in Table 4.

[0041] In conjunction with the present invention, the term "Class 3 branching enzyme gene" is to be understood to mean a nucleic acid molecule (cDNA, DNA), which codes a Class 3 branching enzyme, preferably a Class 3 branching enzyme from starch-storing plants, particularly preferably from plant species of the genus *Solanum*, especially preferably from *Solanum tuberosum*.

[0042] A preferred embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant cell or into the genome of the plant.

[0043] In this context, the term "genetic modification" means the introduction of homologous

and/or heterologous foreign nucleic acid molecules into the genome of a plant cell or into the genome of a plant, wherein said introduction of these molecules leads to an increase in the activity of a Class 3 branching enzyme.

[0044] The plant cells according to the invention or plants according to the invention are modified with regard to their genetic information by the introduction of a foreign nucleic acid molecule. The presence or the expression of the foreign nucleic acid molecule leads to a phenotypic change. Here, "phenotypic" change means preferably a measurable change of one or more functions of the cells. For example, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention exhibit an increase in the activity of a Class 3 branching enzyme due to the presence or on the expression of the introduced nucleic acid molecule.

[0045] In conjunction with the present invention, the term "foreign nucleic acid molecule" is understood to mean such a molecule that either does not occur naturally in the corresponding wild type plant cells, or that does not occur naturally in the concrete spatial arrangement in wild type plant cells, or that is localised at a place in the genome of the wild type plant cell at which it does not occur naturally. Preferably, the foreign nucleic acid molecule is a recombinant molecule, which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in vegetable cells. In principle, the foreign nucleic acid molecule can be any nucleic acid molecule, which effects an increase in the activity of a Class 3 branching enzyme in the plant cell or plant.

[0046] In conjunction with the present invention, the term "genome" is to be understood to mean the totality of the genetic material present in a vegetable cell. It is known to the person skilled in the art that, as well as the cell nucleus, other compartments (e.g. plastids, mitochondria) also contain genetic material.

[0047] In a further embodiment, the plant cells according to the invention and the plants according to the invention are characterised in that the foreign nucleic acid molecule codes a Class 3 branching enzyme, preferably a Class 3 branching enzyme from starch-storing plants,

particularly preferably from plants of a species of the genus *Solanum*, especially preferably from *Solanum tuberosum*.

[0048] In a particularly preferred embodiment, the foreign nucleic acid molecule codes a Class 3 branching enzyme with the amino acid sequence specified in SEQ ID NO 4.

[0049] A large number of techniques are available for the introduction of DNA into a vegetable host cell. These techniques include the transformation of vegetable cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation medium, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA by means of the biolistic approach as well as other possibilities. The use of agrobacteria-mediated transformation of plant cells has been intensively investigated and adequately described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblaserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and by An et al. EMBO J. 4, (1985), 277-287. For the transformation of potato, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33, for example.

[0050] The transformation of monocotyledonous plants by means of vectors based on agrobacterium transformation has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al., Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al., Transgenic Res. 2, (1993), 252-265). An alternative system to the transformation of monocotyledonous plants is transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described in the literature many times (cf. e.g. WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

[0051] The successful transformation of other types of cereal has also already been described, for example for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297). All the above methods are suitable within the framework of the present invention.

[0052] Amongst other things, the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells and wild type plants respectively in that they contain a foreign nucleic acid molecule, which does not occur naturally in wild type plant cells or wild type plants, or in that such a molecule is present integrated at a place in the genome of the plant cell according to the invention or in the genome of the plant according to the invention at which it does not occur in wild type plant cells or wild type plants, i.e. in a different genomic environment. Furthermore, plant cells according to the invention and plants according to the invention of this type differ from wild type plant cells and wild type plants respectively in that they contain at least one copy of the foreign nucleic acid molecule stably integrated within their genome, possibly in addition to naturally occurring copies of such a molecule in the wild type plant cells or wild type plants. If the foreign nucleic acid molecule(s) introduced into the plant cells according to the invention or into the plants according to the invention is (are) additional copies of molecules already occurring naturally in the wild type plant cells or wild type plants respectively, then the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively in particular in that this additional copy or these additional copies is (are) localised at places in the genome at which it does not occur (or they do not occur) in wild type plant cells or wild type plants. This can be verified, for example, with the help of a Southern Blot Analysis.

[0053] Furthermore, the plant cells according to the invention and the plants according to the invention can preferably be differentiated from wild type plant cells or wild type plants respectively by at least one of the following characteristics: If the foreign nucleic acid module that has been introduced is heterologous with respect to the plant cell or plant, then the plant cells according to the invention or plants according to the invention have transcripts of the introduced nucleic acid molecules. These can be verified, for example, by Northern blot analysis or by RT-

PCR (Reverse Transcription Polymerase Chain Reaction). Plant cells according to the invention and plants according to the invention, which express an antisense and/or an RNAi transcript, can be verified, for example, with the help of specific nucleic acid probes, which are complementary to the RNA (occurring naturally in the plant cell), which is coding for the protein. Preferably, the plant cells according to the invention and the plants according to the invention contain a protein, which is coded by an introduced nucleic acid molecule. This can be demonstrated by immunological methods, for example, in particular by a Western Blot Analysis.

[0054] If the foreign nucleic acid module that has been introduced is homologous with respect to the plant cell or plant, the plant cells according to the invention or plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively due to the additional expression of the introduced foreign nucleic acid molecule, for example. The plant cells according to the invention and the plants according to the invention preferably contain (sense and/or antisense) transcripts of the foreign nucleic acid molecules. This can be demonstrated by Northern blot analysis, for example, or with the help of so-called quantitative PCR.

[0055] In a special embodiment, the plant cells according to the invention and the plants according to the invention are transgenic plant cells or transgenic plants respectively

[0056] In a further embodiment, the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of

- a) Nucleic acid molecules, which code a protein with the amino acid sequence given under Seq ID NO 4;
- b) Nucleic acid molecules, which code a protein, the amino acid sequence of which has an identity of at least 50% with the amino acid sequence given under SEQ ID NO: 4;
- c) Nucleic acid molecules, which include the nucleotide sequence shown under Seq ID NO 3 or a complementary sequence;
- d) Nucleic acid molecules, the nucleic acid sequence of which has an identity of at least 50% with the nucleic acid sequences described under a) or c);

- e) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a) or c) under stringent conditions;
- f) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), c), d), e) or f) due to the degeneration of the genetic code; and
- g) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e) or f).

[0057] The amino acid sequence specified in SEQ ID NO 4 codes a protein with the activity of a Class 3 branching enzyme from *Solanum tuberosum*.

[0058] A plasmid was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty on 15th September 2003 under the number DSM 15926. The amino acid sequence shown SEQ ID NO 4 can be derived from the coding region of the cDNA sequence integrated in plasmid DSM 15926 and codes for a Class 3 branching enzyme from *Solanum tuberosum*. The present invention relates to nucleic acid molecules, which code a protein with the enzymatic activity of a Class 3 branching enzyme, which includes the amino acid sequence, which is coded by the insertion in plasmid DSM 15926, wherein the coded protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence, which can be derived from the insertion coding a Class 3 branching enzyme in DSM 15926.

[0059] The proteins coded from the different varieties of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example, biological activity, molecular weight, immunological reactivity, conformation, the presence of structural and/or functional domains etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc.

[0060] The molecular weight of the Class 3 branching enzyme from *Solanum tuberosum*

derived from the amino acid sequence shown under SEQ ID NO 4 is ca. 103 kDa. The derived molecular weight of a protein according to the invention therefore preferably lies in the range from 85 kDa to 120 kDa, preferably in the range from 95 kDa to 110 kDa and particularly preferably from ca. kDa 100 to 105 kDa.

[0061] The nucleic acid sequence shown in SEQ ID NO 3 is a cDNA sequence, which includes the coding region for a Class 3 branching enzyme from *Solanum tuberosum*.

[0062] The present invention therefore also relates to nucleic acid molecules, which code a Class 3 branching enzyme and the coding region of the nucleotide sequence shown under Seq ID NO 3 or a complimentary sequence, nucleic acid molecules, which include the coding region of the nucleotide sequence of the insertion contained in plasmid DSM 15926, and nucleic acid molecules, which have an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of at least 95% with the said nucleic acid molecules.

[0063] With the help of the sequence information of the nucleic acid molecule according to the invention or with the help of the nucleic acid molecule according to the invention, it is now possible for the person skilled in the art to isolate homologous sequences from other plant species, preferably from starch-storing plants, preferably from plant species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*. This can be carried out, for example, with the help of conventional methods such as the examination of cDNA or genomic banks with suitable hybridisation samples. The person skilled in the art knows that homologous sequences can also be isolated with the help of (degenerated) oligonucleotides and the use of PCR-based methods.

[0064] The examination of databases, such as are made available, for example, by EMBL (<http://www.ebi.ac.uk/Tools/index.htm>) or NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), can also be used for identifying homologous sequences, which code for a Class 3 branching enzyme. In this case, one or more sequences are specified as a so-called query. This query sequence is then compared by means of statistical

computer programs with sequences, which are contained in the selected databases. Such database queries (e.g. blast or fasta searches) are known to the person skilled in the art and can be carried out by various providers. If such a database query is carried out, e.g. at the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), then the standard settings, which are specified for the particular comparison inquiry, should be used. For protein sequence comparisons (blastp), these are the following settings: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1.

[0065] For nucleic acid sequence comparisons (blastn), the following parameters must be set: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 11.

[0066] With such a database search, the sequences described in the present invention can be used as a query sequence in order to identify further nucleic acid molecules and/or proteins, which code a Class 3 branching enzyme.

[0067] With the help of the described methods, it is also possible to identify and/or isolate nucleic acid molecules according to the invention, which hybridise with the sequence specified under SEQ ID NO: 3 and which code a Class 3 branching enzyme.

[0068] Within the framework of the present invention, the term "hybridising" means hybridisation under conventional hybridisation conditions, preferably under stringent conditions such as, for example, are described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Particularly preferably, "hybridising" means hybridisation under the following conditions:

[0069] Hybridisation buffer: 2xSSC; 10xDenhardt solution (Ficoll 400+PEG+BSA; Ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na₂HPO₄; 250 µg/ml herring sperm DNA; 50 µg/ml tRNA; or 25 M sodium phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS

[0070] Hybridisation temperature: T=65 to 68°C

[0071] Wash buffer: 0.2xSSC; 0.1% SDS

[0072] Wash temperature: T=65 to 68°C.

[0073] In principle, nucleic acid molecules, which hybridise with the nucleic acid molecules according to the invention, can originate from any plant species, which expresses an appropriate protein, preferably they originate from starch-storing plants, preferably from species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*. Nucleic acid molecules, which hybridise with the molecules according to the invention, can, for example, be isolated from genomic or from cDNA libraries. The identification and isolation of nuclear acid molecules of this type can be carried out using the nucleic acid molecules according to the invention or parts of these molecules or the reverse complements of these molecules, e.g. by means of hybridisation according to standard methods (see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) or by amplification using PCR.

[0074] Nucleic acid molecules, which exactly or essentially have the nucleotide sequence specified under SEQ ID NO: 3, or parts of this sequence, can be used as hybridisation samples. The fragments used as hybridisation samples can also be synthetic fragments or oligonucleotides, which have been manufactured using established synthesising techniques and the sequence of which corresponds essentially with that of a nucleic acid molecule according to the invention. If genes have been identified and isolated, which hybridise with the nucleic acid sequences according to the invention, then a determination of this sequence and an analysis of the characteristics of the proteins coded by this sequence should be carried out in order to establish whether a Class 3 branching enzyme is involved. Homology comparisons on the level of the nucleic acid or amino acid sequence and a determination of the enzymatic activity are particularly suitable for this purpose. As described above, the activity of a Class 3 branching enzyme can take place by expression in *E. coli* strains, which themselves do not express an active branching enzyme (Kiel et al., 1987 Mol. Gen. Genet. 207: 294-301); Guan et al., 1995, Proc. Natl. Acad. Sci. 92, 964-967).

[0075] The molecules hybridising with the nucleic acid molecules according to the invention particularly include fragments, derivatives and allelic variants of the nucleic acid molecules described above, which code a Class 3 branching enzyme from plants, preferably from starch-storing plants, preferably from plant species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*. In conjunction with the present invention, the term "derivative" means that the sequences of these molecules differ at one or more positions from the sequences of the nucleic acid molecules described above and have a high degree of identity with these sequences. Here, the deviation from the nucleic acid molecules described above can have come about, for example, due to deletion, addition, substitution, insertion or recombination.

[0076] The proteins coded from the different derivatives of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example, biological activity, substrate specificity, molecular weight, immunological reactivity, conformation, the presence of structural and/or functional domains etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc.

[0077] In conjunction with the present invention, the term "identity" means a sequence identity over the whole length of the coding region of at least 60%, in particular an identity of at least 70%, preferably greater than 80%, particularly preferably greater than 90% and especially of at least 95%. In conjunction with the present invention, the term "identity" is to be understood to mean the number of amino acids/nucleotides (identity) corresponding with other proteins/nucleic acids, expressed as a percentage. Identity is preferably determined by comparing the Seq. ID NO 4 or SEQ ID NO 3 with other proteins/nucleic acids with the help of computer programs. If sequences that are compared with one another have different lengths, the identity is to be determined in such a way that the number of amino acids, which have the shorter sequence in common with the longer sequence, determines the percentage quotient of the identity. Preferably, identity is determined by means of the computer program ClustalW, which is well known and available to the public (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680).

ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can also be downloaded from different Internet sites, including the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; <ftp://ftp-igbmc.u-strasbg.fr/pub/>) and the EBI (<ftp://ftp.ebi.ac.uk/pub/software/>) as well as from all mirrored Internet sites of the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

[0078] Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between proteins according to the invention and other proteins. In doing so, the following parameters must be set: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

[0079] Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between the nucleotide sequence of the nucleic acid molecules according to the invention, for example, and the nucleotide sequence of other nucleic acid molecules. In doing so, the following parameters must be set: KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

[0080] Furthermore, identity means that functional and/or structural equivalence exists between the nucleic acid molecules concerned or the proteins coded by them. The nucleic acid molecules, which are homologous to the molecules described above and constitute derivatives of these molecules, are generally variations of these molecules, which constitute modifications, which execute the same biological function. At the same time, the variations can occur naturally, for example they can be sequences from other plant species, or they can be mutations, wherein these mutations may have occurred in a natural manner or have been introduced by objective mutagenesis. The variations can also be synthetically manufactured sequences. The allelic variants can be both naturally occurring variants and also synthetically manufactured variants or variants produced by recombinant DNA techniques. Nucleic acid molecules, which deviate from

nucleic acid molecules according to the invention due to degeneration of the genetic code, constitute a special form of derivatives.

[0081] The nucleic acid molecules according to the invention can be any nucleic acid molecules, in particular DNA or RNA molecules, for example cDNA, genomic DNA, mRNA etc. They can be naturally occurring molecules or molecules manufactured by genetic or chemical synthesis methods. They can be single-stranded molecules, which either contain the coding or the non-coding strand, or double-stranded molecules.

[0082] Furthermore, the present invention relates to nucleic acid molecules of at least 21, preferably more than 50 and particularly preferably more than 200 nucleotides length, which specifically hybridise with at least one nucleic acid molecule according to the invention. Here, specifically hybridise means that these molecules hybridise with nucleic acid molecules, which code a protein according to the invention, but not with nucleic acid molecules, which code other proteins.

[0083] A further embodiment of the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of

- a) T-DNA molecules, which lead to an increase in the expression of a Class 3 branching enzyme gene due to integration in the vegetable genome (T-DNA activation tagging);
- b) DNA molecules, which contain transposons, which lead to an increase in the expression of a Class 3 branching enzyme gene due to integration in the vegetable genome (transposon activation tagging);
- c) DNA molecules, which code a Class 3 branching enzyme and which are linked with regulatory sequences, which guarantee transcription in vegetable cells and lead to an increase in Class 3 branching enzyme activity in the cell.
- d) Nucleic acid molecules introduced by means of in vivo mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding a Class 3 branching enzyme, wherein the mutation or insertion effects an increase in the expression of a gene coding a Class 3 branching enzyme.

[0084] In conjunction with the present invention, plant cells according to the invention and plants according to the invention can also be manufactured by the use of so-called insertion mutagenesis (overview article: Thorneycroft et al., 2001, *Journal of experimental Botany* 52 (361), 1593-1601). In conjunction with the present invention, insertion mutagenesis is to be understood to mean particularly the insertion of transposons or so-called transfer DNA (T-DNA) into a gene or in the vicinity of a gene coding for a Class 3 branching enzyme, whereby as a result of which the activity of a Class 3 branching enzyme in the cell concerned is increased.

[0085] The transposons can be both those that occur naturally in the cell (endogenous transposons) and also those that do not occur naturally in said cell but are introduced into the cell (heterologous transposons) by means of genetic engineering methods, such as transformation of the cell, for example. Changing the expression of genes by means of transposons is known to the person skilled in the art. An overview of the use of endogenous and heterologous transposons as tools in plant biotechnology is presented in Ramachandran and Sundaresan (2001, *Plant Physiology and Biochemistry* 39, 234-252).

[0086] T-DNA insertion mutagenesis is based on the fact that certain sections (T-DNA) of Ti plasmids from *Agrobacterium* can integrate into the genome of vegetable cells. The place of integration in the vegetable chromosome is not defined, but can take place at any point. If the T-DNA integrates into a part of the chromosome or in the vicinity of a part of the chromosome, which constitutes a gene function, then this can lead to an increase in the gene expression and thus also to a change in the activity of a protein coded by the gene concerned. Here, the sequences inserted into the genome (in particular transposons or T-DNA) are distinguished by the fact that they contain sequences, which lead to an activation of regulatory sequences of a Class 3 branching enzyme gene ("activation tagging").

[0087] Plant cells and plants according to the invention can be produced by means of the so-called "activation tagging" method (see, for example, Walden et al., *Plant J.* (1991), 281-288; Walden et al., *Plant Mol. Biol.* 26 (1994), 1521-1528). These methods are based on activating endogenous promoters by means of "enhancer" sequences, such as the enhancer of the 35S RNA promoter of the cauliflower mosaic virus, or the octopine synthase enhancer.

[0088] In conjunction with the present invention, the term "T-DNA activation tagging" is to be understood to mean a T-DNA fragment, which contains "enhancer" sequences and which leads to an increase in the activity of at least one Class 3 branching enzyme by integration into the genome of a plant cell.

[0089] In conjunction with the present invention, the term "transposon activation tagging" is to be understood to mean a transposon, which contains "enhancer" sequences and which leads to an increase in the activity of at least one Class 3 branching enzyme by integration into the genome of a plant cell.

[0090] In a preferred embodiment, the DNA molecules according to the invention, which code a Class 3 branching enzyme, are linked with regulatory sequences, which guarantee transcription in vegetable cells and lead to an increase in Class 3 branching enzyme activity in the cell. In this case, the nucleic acid molecules according to the invention are present in "sense" orientation to the regulatory sequences.

[0091] For expressing nucleic acid molecules according to the invention, which code Class 3 branching enzymes, these are preferably linked with regulatory DNA sequences, which guarantee transcription in vegetable cells. In particular, these include promoters. In general, any promoter that is active in vegetable cells is eligible for expression. At the same time, the promoter can be chosen so that expression takes place constitutively or only in a certain tissue, at a certain stage of the plant development or at a time determined by external influences. The promoter can be homologous or heterologous both with respect to the plant and with respect to the nucleic acid molecule.

[0092] Suitable promoters are, for example, the promoter of the 35S RNA of the cauliflower mosaic virus and the ubiquitin promoter from maize for constitutive expression, the patatin promoter B33 (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29) for tuber-specific expression in potatoes or a promoter, which only ensures expression in photosynthetically active tissues, e.g. the ST-LS1 promoter (Stockhaus et al., Proc. Natl. Acad. Sci. USA 84 (1987), 7943-7947;

Stockhaus et al., EMBO J. 8 (1989), 2445-2451) or, for endosperm-specific expression of the HMG promoter from wheat, the USP promoter, the phaseolin promoter, promoters of zein genes from maize (Pedersen et al., Cell 29 (1982), 1015-1026; Quattroccio et al., Plant Mol. Biol. 15 (1990), 81-93), glutelin promoter (Leisy et al., Plant Mol. Biol. 14 (1990), 41-50; Zheng et al., Plant J. 4 (1993), 357-366; Yoshihara et al., FEBS Lett. 383 (1996), 213-218) or shrunken-1 promoter (Werr et al., EMBO J. 4 (1985), 1373-1380). However, promoters can also be used, which are only activated at a time determined by external influences (see for example WO 9307279). Promoters of heat-shock proteins, which allow simple induction, can be of particular interest here. Furthermore, seed-specific promoters can be used, such as the USP promoter from *Vicia faba*, which guarantees seed-specific expression in *Vicia faba* and other plants (Fiedler et al., Plant Mol. Biol. 22 (1993), 669-679; Bäumlein et al., Mol. Gen. Genet. 225 (1991), 459-467).

[0093] Furthermore, a termination sequence (polyadenylation signal) can be present, which is used for adding a poly-A tail to the transcript. A function in the stabilisation of the transcripts is ascribed to the poly-A tail. Elements of this type are described in the literature (cf. Gielen et al., EMBO J. 8 (1989), 23-29) and can be exchanged at will.

[0094] Furthermore, plant cells according to the invention and plants according to the invention can be manufactured by means of so-called "in situ activation". In this case, the introduced genetic modification effects a change in the regulatory sequences of endogenous Class 3 branching enzyme genes, which leads to an increased expression of Class 3 branching enzyme genes. Preferably, the activation of a Class 3 branching enzyme gene takes place by "in vivo" mutagenesis of a promoter or of "enhancer" sequences of an endogenous Class 3 branching enzyme gene. In doing so, a promoter or an "enhancer" sequence, for example, can be changed in such a way that the mutation produced leads to an increased expression of a Class 3 branching enzyme gene in plant cells according to the invention or plants according to the invention in comparison with the expression of a Class 3 branching enzyme gene in wild type plant cells or wild type plants. The mutation in a promoter or an "enhancer" sequence can also lead to Class 3 branching enzyme genes in plant cells according to the invention or plants according to the invention being expressed at a time at which they would not be expressed in wild type plant cells or wild type plants.

[0095] In so-called "in vivo mutagenesis", a hybrid RNA-DNA oligonucleotide ("Chimeroplast") is introduced into plant cells (Kipp, P.B. et al., Poster Session at the "5th International Congress of Plant Molecular Biology, 21st-27th September 1997, Singapore; R. A. Dixon and C.J. Arntzen, meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15, (1997), 441-447; international patent application WO 9515972; Kren et al., Hepatology 25, (1997), 1462-1468; Cole-Strauss et al., Science 273, (1996), 1386-1389; Beetham et al., 1999, PNAS 96, 8774-8778).

[0096] A part of the DNA components of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous Class 3 branching enzyme gene, but, in comparison with the nucleic acid sequence of a Class 3 branching enzyme gene, it has a mutation or contains a heterologous region, which is surrounded by the homologous regions.

[0097] By base pairing of the homologous regions of the RNA-DNA oligonucleotide and the endogenous nucleic acid molecule followed by homologous recombination, the mutation or heterologous region contained in the DNA components of the RNA-DNA oligonucleotide can be transferred into the genome of a plant cell. This leads to an increase in the activity of one or more Class 3 branching enzymes.

[0098] All these methods are based on the introduction of a foreign nucleic acid molecule into the genome of a plant cell or plant and are therefore basically suitable for the manufacture of plant cells according to the invention and plants according to the invention.

[0099] Surprisingly, it has been found that plant cells according to the invention and plants according to the invention synthesise a modified starch in comparison with starch of corresponding wild type plant cells or wild type plants that have not been genetically modified.

[00100] The plant cells according to the invention and plants according to the invention synthesise a modified starch, which in its physical-chemical characteristics, in particular the amylose content or the amylose/amyllopectin ratio, the degree of branching, the average chain

length, the side chain distribution, the viscosity behaviour, the gelling strength, the starch grain size and/or the starch grain morphology, is changed in comparison with the synthesised starch in wild type plant cells or wild type plants, so that this is better suited for special applications.

[00101] The present invention therefore also includes plant cells according to the invention and plants according to the invention, which synthesise a modified starch.

[00102] In conjunction with the present invention, the term "modified starch" means that the starch has changed physical-chemical characteristics compared with non-modified starch obtainable from corresponding wild type plant cells or wild type plants.

[00103] In a preferred embodiment of the present invention, the modified starch is native starch.

[00104] In conjunction with the present invention, the term "native starch" means that the starch is isolated from plants according to the invention, harvestable plant plants according to the invention or propagation material of plants according to the invention by methods known to the person skilled in the art.

[00105] Furthermore, genetically modified plants, which contain the plant cells according to the invention, are also the subject matter of the invention. Plants of this type can be produced from plant cells according to the invention by regeneration.

[00106] In principle, the plants according to the invention can be plants of any plant species, i.e. both monocotyledonous and dicotyledonous plants. Preferably they are useful plants, i.e. plants, which are cultivated by people for the purposes of food or for technical, in particular industrial purposes.

[00107] In a further preferred embodiment, the plant according to the invention is a starch-storing plant.

[00108] In a further preferred embodiment, the present invention relates to starch-storing

plants according to the invention of the genus *Solanum*, in particular *Solanum tuberosum*.

[00109] The term "starch-storing plants" includes all plants with starch-storing plant parts such as, for example, maize, rice, wheat, rye, oat, barley, cassava, potato, sago, mung bean, pea or sorghum. Preferred starch-storing plant parts are, for example, tubers, storage roots and grains containing an endosperm; tubers are particularly preferred; tubers of potato plants are especially preferred.

[00110] In conjunction with the present invention, the term "potato plant" or "potato" means plant species of the genus *Solanum*, in particular tuber-producing species of the genus *Solanum* and especially *Solanum tuberosum*.

[00111] The present invention also relates to propagation material of plants according to the invention containing a plant cell according to the invention.

[00112] Here, the term "propagation material" includes those constituents of the plant that are suitable for producing offspring by vegetative or sexual means. Cuttings, callus cultures, rhizomes or tubers, for example, are suitable for vegetative propagation. Other propagation material includes, for example, fruits, seeds, seedlings, protoplasts, cell cultures, etc. Preferably, the propagation material is seeds and particularly preferably tubers.

[00113] In a further embodiment, the present invention relates to harvestable plant parts of plants according to the invention such as fruits, storage roots, roots, blooms, buds, shoots or stems, preferably seeds or tubers, wherein these harvestable parts contain plant cells according to the invention.

[00114] Furthermore, the present invention also relates to a method for the manufacture of a genetically modified plant according to the invention, wherein

a) a plant cell is genetically modified, whereby the genetic modification leads to an increase in the activity of a Class 3 vegetable branching enzyme in comparison with corresponding wild type plant cells that have not been genetically modified;

- b) a plant is regenerated from plant cells from Step a); and
- c) if necessary, further plants are produced with the help of the plants according to Step b).

[00115] The genetic modification introduced into the plant cell according to Step a) can basically be any type of modification, which leads to the reduction [sic?] of the activity of a Class 3 branching enzyme. The regeneration of the plants according to Step (b) can be carried out using methods known to the person skilled in the art (e.g. described in "Plant Cell Culture Protocols", 1999, edt. by R.D. Hall, Humana Press, ISBN 0-89603-549-2).

[00116] The production of further plants according to Step (c) of the method according to the invention can be carried out, for example, by vegetative propagation (for example using cuttings, tubers or by means of callus culture and regeneration of whole plants) or by sexual propagation. Here, sexual propagation preferably takes place under controlled conditions, i.e. selected plants with particular characteristics are crossed and propagated with one another.

[00117] In a preferred embodiment of the method according to the invention, the genetic modification consists in the introduction of a foreign nucleic acid molecule into the genome of the plant cell, wherein the presence or the expression of said foreign nucleic acid molecule leads to an increased activity of a Class 3 branching enzyme in the cell.

[00118] In a further preferred embodiment, the method according to the invention is used for producing potato plants according to the invention.

[00119] In a further preferred embodiment of the method according to the invention, the foreign nucleic acid molecule is chosen from the group consisting of

- a) Nucleic acid molecules, which code a protein with the amino acid sequence given under Seq ID NO 4;
- b) Nucleic acid molecules, which code a protein, the amino acid sequence of which has an identity of at least 50% with the amino acid sequence given under SEQ ID NO: 4;
- c) Nucleic acid molecules, which include the nucleotide sequence shown under Seq ID

NO 3 or a complimentary sequence;

- d) Nucleic acid molecules, the nucleic acid sequence of which has an identity of at least 50% with the nucleic acid sequences described under a) or c);
- e) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a) or c) under stringent conditions;
- f) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), c), d), e) or f) due to the degeneration of the genetic code; and
- g) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e) or f).

[00120] In a further preferred embodiment of the method according to the invention, the foreign nucleic acid molecule is chosen from the group consisting of

- a) T-DNA molecules, which lead to an increase in the expression of a Class 3 branching enzyme gene due to integration in the vegetable genome (T-DNA activation tagging);
- b) DNA molecules, which contain transposons, which lead to an increase in the expression of a Class 3 branching enzyme gene due to integration in the vegetable genome (transposon activation tagging);
- c) DNA molecules, which code a Class 3 branching enzyme and which are linked with regulatory sequences, which guarantee transcription in vegetable cells and lead to an increase in Class 3 branching enzyme activity in the cell;
- d) Nucleic acid molecules introduced by means of in vivo mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding a Class 3 branching enzyme, wherein the mutation or insertion effects an increase in the expression of a gene coding a Class 3 branching enzyme.

[00121] The present invention also relates to the plants obtained by the method according to the invention.

[00122] In a further embodiment of the method according to the invention, the plants according to the invention synthesise a modified starch in comparison with corresponding wild

type plants that have not been genetically modified.

[00123] Surprisingly, it has been found that plant cells and plants, which have an increased activity of a Class 3 branching enzyme, synthesise a modified starch.

[00124] The present invention also relates to the plants obtained by the method according to the invention.

[00125] The present invention also relates to modified starches obtainable from plant cells according to the invention or plants according to the invention, from propagation material according to the invention or from harvestable plant parts according to the invention.

[00126] In a particularly preferred embodiment, the present invention relates to modified potato starch.

[00127] Furthermore the present invention relates to a method for the manufacture of a modified starch including the step of extracting the starch from a plant cell according to the invention or from a plant according to the invention, from propagation material according to the invention of such a plant and/or from harvestable plant parts according to the invention of such a plant, preferably from starch-storing parts according to the invention of a plant. Preferably, such a method also includes the step of harvesting the cultivated plants or plant parts and/or the propagation material of these plants before the extraction of the starch and, further, particularly preferably the step of cultivating plants according to the invention before harvesting.

[00128] Methods for extracting starches from plants or from starch-storing parts of plants are known to the person skilled in the art. Furthermore, methods for extracting starch from different starch-storing plants are described, e.g. in Starch: Chemistry and Technology (Publisher: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see e.g. Chapter XII, Page 412-468: Maize and Sorghum Starches: Manufacture; by Watson; Chapter XIII, Page 469-479: Tapioca, Arrowroot and Sago Starches: Manufacture; by Corbishley and Miller; Chapter XIV, Page 479-490: Potato starch: Manufacture and Uses; by Mitch; Chapter XV, Page 491 to 506: Wheat starch: Manufacture, Modification and Uses; by

Knight and Oson; and Chapter XVI, Page 507 to 528: Rice starch: Manufacture and Uses; by Rohmer and Klem; Maize starch: Eckhoff et al., Cereal Chem. 73 (1996), 54-57, the extraction of maize starch on an industrial scale is generally achieved by so-called "wet milling"). Devices, which are in common use in methods for extracting starch from plant material are separators, decanters, hydrocyclones, spray dryers and fluid bed dryers.

[00129] In conjunction with the present invention, the term "starch-storing parts" is to be understood to mean such parts of a plant in which, in contrast to transitory leaf starch, starch is stored as a deposit for surviving for longer periods. Preferred starch-storing parts are tubers, storage roots, seeds or endosperm; particularly preferred are potato tubers or the endospermo of maize, wheat or rice plants.

[00130] Modified starch obtainable by a method according to the invention for manufacturing modified starch is also the subject matter of the present invention.

[00131] Furthermore, the use of plant cells according to the invention or plants according to the invention for manufacturing a modified starch are the subject matter of the present invention.

[00132] The person skilled in the art knows that the characteristics of starch can be changed by thermal, chemical, enzymatic or mechanical derivation, for example. Derived starches are particularly suitable for different applications in the foodstuffs and/or non-foodstuffs sector. The starches according to the invention are better suited as a starting substance for the manufacture of derived starches than conventional starches.

[00133] The present invention therefore also relates to the manufacture of a derived starch, wherein modified starch according to the invention is derived retrospectively.

[00134] In conjunction with the present invention, the term "derived starch" is to be understood to mean a modified starch according to the invention, the characteristics of which have been changed after isolation from vegetable cells with the help of chemical, enzymatic, thermal or mechanical methods. In a preferred embodiment of the present invention, the derived

starch according to the invention is starch that has been heat-treated and/or acid-treated. In a further preferred embodiment, the derived starches are starch ethers, in particular starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxymethyl ethers, nitrogen-containing starch ethers, phosphate-containing starch ethers or sulphur-containing starch ethers. In a further preferred embodiment, the derived starches are cross-linked starches. In a further preferred embodiment, the derived starches are starch graft polymers. In a further preferred embodiment, the derived starches are oxidised starches. In a further preferred embodiment, the derived starches are starch esters, in particular starch esters, which have been introduced into the starch using organic acids. Particularly preferably these are phosphate, nitrate, sulphate, xanthate, acetate or citrate starches.

[00135] The derived starches according to the invention are suitable for different applications in the foodstuffs and/or non-foodstuffs sector. Methods for manufacturing derived starches according to the invention are known to the person skilled in the art and are adequately described in the general literature. An overview on the manufacture of derived starches can be found, for example, in Orthoefer (in Corn, Chemistry and Technology, 1987, eds. Watson und Ramstad, Chapter 16, 479-499).

[00136] Derived starch obtainable by the method according to the invention for manufacturing a derived starch is also the subject matter of the present invention.

[00137] Furthermore, the use of modified starches according to the invention for manufacturing derived starch is the subject matter of the present invention.

Description of sequences

[00138] SEQ ID NO 1: Nucleic acid sequence containing the coding region of the 3'-area of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence is inserted in plasmid AN 46-196.

[00139] SEQ ID NO 2: Nucleic acid sequence containing the coding region of the 5'-area of a

Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence is inserted in plasmid AN 47-196.

[00140] SEQ ID NO 3: Nucleic acid sequence containing the full coding region of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence is inserted in plasmid AN 49 and was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty on 15th September 2003 under the number DSM 15926.

[00141] SEQ ID NO 4: Amino acid sequence coding a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence can be derived from the nucleic acid sequence inserted in plasmid AN 49 or from the nucleic acid sequence described under SEQ ID NO 3.

[00142] SEQ ID NO 5: Nucleic acid sequence containing the full coding region of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence has been obtained by combining the nucleic acid sequences described under SEQ ID NO 1 and SEQ ID NO 2. This nucleic acid sequence constitutes an allelic variant of the nucleic acid sequence described under SEQ ID NO 3 coding a Class 3 branching enzyme.

[00143] SEQ ID NO 6: Amino acid sequence coding a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence can be derived from the nucleic acid sequence described under SEQ ID NO 5 and constitutes an allelic variant of the amino acid sequence described under SEQ ID NO 4 coding a Class 3 branching enzyme.

Description of the figures

[00144] Fig. 1 shows the relative amount of mRNA in seeds of genetically modified rice plants (GOAS0453), expressing a potato branching enzyme Class 3. Also shown is the example of a genetically non modified wild type plant (wt).

General methods

[00145] The following methods were used in the examples:

1. Demonstration of the activity of a Class 3 branching enzyme

[00146] The activity of a Class 3 branching enzyme was demonstrated with the help of non-denaturing gel electrophoresis as follows:

[00147] To isolate proteins from plants, the test material was ground with a pestle in liquid nitrogen, absorbed into an extraction buffer (50 mM Na citrate, pH 6.5; 1 mM EDTA, 4 mM DTT) and, after centrifugation (10 min, 14.000 g, 4 °C), was used directly for measurement of the protein content according to Bradford. Subsequently, 5µg to 20 µg total protein extract was mixed with 4X loading buffer (20% glycerol, 125 mM Tris HCl, pH 6.8) and loaded onto a BE activity gel. The BE activity gel was made up as follows: 2.5 ml 30% acrylamide:0.8% bisacrylamide, 0.1 ml running buffer, 7.4 ml H₂O, 10% ammonium persulphate solution and 5 µl N,N,N',N'- tetramethylethylenediamine (TEMED). The running buffer (RB) was made up as follows: RB = 30.2 g Tris base, pH 8.0, 144 g glycine on 1 L H₂O. On completion of the gel run, each of the gels was incubated overnight at 37 °C in 25 ml "phosphorylase buffer" (25 ml 1M Na citrate pH 7.0, 0.47 g glucose-1-phosphate, 12.5 mg AMP, 2.5 mg phosphorylase a/b from "rabbit"). The gels were coloured with Lugol's solution.

2. Transformation of *Oryza sativa* (cv. M202)

[00148] Rice plants were transformed in accordance with the methods described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

3. Starch analysis

a) Determination of the amylose content and of the amylose/amyllopectin ratio

[00149] Rice flour was obtained from polished rice by milling the grains in a Cyclotec labmill to particle size smaller than 0,5 mm.

[00150] Amylose content was determined by the method of Juliano.(1971)(Cereal Science Today, 16, p334-340) with slight modifications. 50mg of rice flour are weighed in a 100ml

Erlenmeyer flask and wetted with 1 ml of 95% ethanol to avoid lumping. After addition of 9 ml of 1 M NaOH the sample is heated to 100°C for 20 min, cooled to room temperature and filled up to 100 ml with distilled water.

[00151] An aliquot of 100 μ l is mixed with 10 μ l 1M acetic acid, 20 μ l Lugol solution (0,2% I₂ and 2% KI) and 970 μ l water. After 10 minutes the OD at 620 nm is determined. The amylose content is calculated from a standard curve generated from samples with defined amount of potato amylose.

b) Determination of the phosphate content

[00152] In starch, the positions C2, C3 and C6 of the glucose units can be phosphorylated. Determination of C6-P content of rice starch is performed either on single mature grains hydrolysed in 200 μ l of 0,7 M HCl or on 50 mg rice flour hydrolysed in 500 μ l 0,7 M HCl. After 4 h hydrolysis at 95°C under continuous shaking, samples are centrifuged for 10 minutes at 15500xg and the supernatants are applied to a spin module containing 0,2 μ m PTFE-filter membran for complete clarification of the hydrolyzate (5 min 1000xg). 20 μ l of the supernatants are mixed with 180 μ l of imidazole buffer (300 mM imidazole, pH 7.2; 7,5 mM MgCl₂, 1 mM EDTA and 0.4 mM NADP). The measurement was carried out in a photometer at 340 nm. After the base absorption had been established, the enzyme reaction was started by addition of 0,4 units glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption is directly proportional to the concentration of the G-6-P content of the starch.

[00153] The total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118).

[00154] Approximately 50 mg of starch are treated with 30 μ l of ethanolic magnesium nitrate solution and ashed for 3 hours at 500°C in a muffle oven. The residue is treated with 300 μ l of 0.5 M hydrochloric acid and incubated for 30 minutes at 60°C. One aliquot is subsequently made up to 300 μ l 0.5 M hydrochloric acid and this is added to a mixture of 100 μ l of 10% ascorbic acid and 600 μ l of 0.42% ammonium molybdate in 2 M sulphuric acid and incubated for 20 minutes at

45°C.

[00155] This is followed by a photometric determination at 820 nm with a phosphate calibration series as standard.

c) Determination of the viscosity characteristics by means of a Rapid Visco Analyser (RVA)

[00156] 3 g of rice flour (DM) are taken up in 25 ml of H₂O (VE-type water, conductivity of at least 15 mega ohm) and used for the analysis in a Rapid Visco Analyser Super3 (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The apparatus is operated following the manufacturer's instructions. The viscosity values are indicated in Centipoise (cP) in accordance with the manufacturer's operating manual, which is incorporated into the description herewith by reference. To determine the viscosity of the aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for a minute (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 12°C per minute (step 2). The temperature is held for 2.5 minutes at 95°C (step 3) and then cooled from 95°C to 50°C at 12°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 2 minutes. The viscosity is determined during the entire duration.

[00157] After the program has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis after 24 hours incubation at room temperature.

[00158] The profile of the RVA analysis contains parameters which are shown for the comparison of different measurements and substances. In the context of the present invention, the following terms are to be understood as follows:

1. Maximum viscosity (RVA Max)

[00159] The maximum viscosity is understood as meaning the highest viscosity value, measured in cP, obtained in step 2 or 3 of the temperature profile.

2. Minimum viscosity (RVA Min)

[00160] The minimum viscosity is understood as meaning the lowest viscosity value, measured in cP, observed in the temperature profile after the maximum viscosity. Normally, this takes place in step 3 of the temperature profile.

3. Final viscosity (RVA Fin)

[00161] The final viscosity is understood as meaning the viscosity value, measured in cP, observed at the end of the measurement.

4. Setback (RVA Set)

[00162] What is known as the "setback" is calculated by subtracting the value of the final viscosity from that of the minimum occurring after the maximum viscosity in the curve.

5. Gelatinization temperature (RVA PT)

[00163] The gelatinization temperature is understood as meaning the point in time of the temperature profile where, for the first time, the viscosity increases drastically for a brief period.

d) Determination of the gel strength (Texture Analyser)

[00164] 3 g of rice flour (DM) are gelatinized in the RVA apparatus in 25 ml of an aqueous suspension (temperature program: see item c) "Determination of the viscosity characteristics by means of a Rapid Visco Analyser (RVA)" and subsequently stored for 24 hours at room temperature in a sealed container. The samples are fixed under the probe (round piston with planar surface) of a Texture Analyser TA-XT2 from Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following parameters:

| | | |
|---|----------------------|---------------------|
| - | Test speed | 0.5 mm/s |
| - | Depth of penetration | 7 mm |
| - | Contact surface | 113 mm ² |
| - | Pressure | 2 g |

e) Analysis of the side-chain distribution of the amylopectin by means of ion-exchange chromatography

Definition Pfam Isoamylase N Domain

Annex 1

Pfam Entity Isoamylase_N

Accession number: PF02922

Previous identifiers: isoamylase_N;

Isoamylase N-terminal domain

This domain is found in a range of enzymes that act on branched substrates - isoamylase, pullulanase and branching enzyme. This family also contains the beta subunit of 5' AMP activated kinase. This family forms **interactions** with other Pfam families, to view them [click here](#)

INTERPRO description (entry IPR004193)

O-Glycosyl hydrolases (EC 3.2.1.-) are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. A classification system for glycosyl hydrolases, based on sequence similarity, has led to the definition of 85 different families [PUBMED:7624375](#), [PUBMED:8535779](#), [PUBMED](#). This classification is available on the CAZy (CAbohydrate-Active Enzymes) web site [PUBMED](#). Because the fold of proteins is better conserved than their sequences, some of the families can be grouped in 'clans'.

Enzymes containing this domain belong to family 13 () of the glycosyl hydrolases. This domain is found in a range of enzymes that act on branched substrates ie, isoamylase, pullulanase and branching enzyme. Isoamylase hydrolyses 1,6-alpha-D-glucosidic branch linkages in glycogen, amylopectin and dextrin; 1,4-alpha-glucan branching enzyme functions in the formation of 1,6-glucosidic linkages of glycogen; and pullulanase is a starch-debranching enzyme.

QuickGO

| Key: | Domain | Chain | Start | End | Residue | Residue |
|---------------------|----------|----------|-----------|------------|---------|---------|
| Isoamylase_N | N | A | 74 | 147 | | |
| Isoamylase_N | N | B | 74 | 147 | | |
| Isoamylase_N | N | C | 74 | 147 | | |

Figure 1: 1z0m SUGAR BINDING PROTEIN THE GLYCOGEN-BINDING DOMAIN OF THE AMP-ACTIVATED PROTEIN KINASE BETA1 SUBUNIT

Key:

| Domain | Start | End | Residue | Residue |
|---------------------|----------|-----------|------------|---------|
| Isoamylase_N | A | 74 | 147 | |
| Isoamylase_N | B | 74 | 147 | |
| Isoamylase_N | C | 74 | 147 | |

Function

hydrolyase activity, hydrolyzing O-glycosyl compounds (GO:00004553)

Process

carbohydrate metabolism (GO:0005925)

The Swissprot/PDB mapping was provided by [MSD](#)

Alignment

Domain organisation

1b12

Seed (60) Full (1021)

Format: Coloured alignment Black & white

Further alignment options [here](#)
Help relating to Pfam alignments [here](#)

Species Distribution

View alignments & domain organisation by species Show all levels

Tree depth: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1041 1042 1043 1044 1045 1046 1047 1048 1049 1050 1051 1052 1053 1054 1055 1056 1057 1058 1059 1060 1061 1062 1063 1064 1065 1066 1067 1068 1069 1070 1071 1072 1073 1074 1075 1076 1077 1078 1079 1080 1081 1082 1083 1084 1085 1086 1087 1088 1089 1090 1091 1092 1093 1094 1095 1096 1097 1098 1099 1100 1101 1102 1103 1104 1105 1106 1107 1108 1109 1110 1111 1112 1113 1114 1115 1116 1117 1118 1119 <input type="checkbox

| Literature References | | Pfam specific information | |
|--|-------------------------------|---|--|
| Author of entry | Griffiths-Jones SR | Type definition | Domain |
| 1. Novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Hudson ER, Pan DA, James J, Luooco JM, Hawley SA, Green KA, Baba O, Terashima T, Hardie DG; Curr Biol 2003;13:861-866. | | Source of seed members | Structural domain |
| | | Average Length | 25 |
| | | Average %id | 12.70 |
| | | Average Coverage | CHANGE% |
| HMMER build information | | | |
| | Pfam_ls [Download HMM] | Pfam_fs [Download HMM] | |
| | Gathering cutoff | -6.5 -6.5; | 16.0 16.0 |
| | Trusted cutoff | -6.5 -6.5; | 16.0 16.0 |
| | Noise cutoff | -6.9 -6.9; | 15.9 15.9 |
| | Build method of HMM | hmmbuild -f HMM_ls SEED hmccalibrate --cpu 1 --seed 0 HMM_ls | hmmbuild -f -F HMM_ls SEED hmccalibrate --cpu 1 --seed 0 HMM_ls |

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Annex 2

Definition of Pfam Alphaamylase Domain



Pfam entry Alpha-amylase

Accession number: PF00128

Previous identifiers: alpha-amylase;

Alpha amylase, catalytic domain

Alpha amylase is classified as family 13 of the glycosyl hydrolases. The structure is an 8 stranded alpha/beta barrel containing the active site, interrupted by a ~70 a.a. calcium-binding domain protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal Greek key beta-barrel domain.

This family forms [Interactions](#) with other Pfam families, to view them click [here](#)

Add Annotation

Figure 1: 1vfk

HYDROLASE
CRYSTAL STRUCTURE OF
THERMOACTINOMYCES VULGARIS R-47
ALPHA- AMYLASE 2-ACARBOSE COMPLEX

Key:

| Domain | Chain | Start | End | Residue | Residue |
|-----------------------------------|-------|-------|-----|---------|---------|
| <u>Alpha-</u> <u>amylase</u> | A | 135 | 494 | | |
| <u>Alpha-</u> <u>amylase</u> N | A | 1 | 117 | | |
| <u>Alpha-</u> <u>amylase</u> B | B | 135 | 494 | | |
| <u>Alpha-</u> <u>amylase</u> N | B | 1 | 117 | | |

Tim barrel glycosyl hydrolase superfamily

This family is a member of the Tim barrel glycosyl hydrolase superfamily clan. This clan includes the following Pfam members:
Glyco_hydro_92; Glyco_hydro_77; Glyco_hydro_70; Glyco_hydro_59; Glyco_hydro_56; Glyco_hydro_53; Glyco_hydro_42; Glyco_hydro_39; Glyco_hydro_31; Glyco_hydro_30; Glyco_hydro_25; Glyco_hydro_20; Glyco_hydro_18; Glyco_hydro_17; Glyco_hydro_14; Glyco_hydro_10; Glyco_hydro_11; DUF187; Cellulase; Alpha-amylase; Melibiose;

INTERPRO description (entry IPR006047)

O-Glycosyl hydrolases (EC 3.2.1.) are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. A classification system for glycosyl hydrolases, based on sequence similarity, has led to the definition of 85 different families [PubMed:7624375](#), [PubMed:8535779](#), [PubMed](#). Because the fold of proteins is better conserved than their sequences, some of the families can be grouped in 'clans'. Enzymes containing this domain, such as alpha-amylase, belong to family 13 () of the glycosyl hydrolases. The malto-*genic* alpha-amylase is an enzyme which catalyses hydrolysis of (1-4)-alpha-D-glucosidic linkages in polysaccharides so as to remove successive alpha-maltose residues from the non-reducing ends of the chains in the conversion of starch to maltose. Other enzymes include neopullulanase, which hydrolyses pullulan to panose, and cyclomalto-dextrinase, which hydrolyses cyclodextrins. This entry represents the catalytic domain found in several protein members of this family. It has a structure consisting of an 8 stranded alpha/beta barrel that contains the active site, interrupted by a ~70 amino acid calcium-binding domain protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal Greek key beta-barrel domain [PubMed:16302977](#).

The SwissprotPDB mapping was provided by MSD

1a47

More information about this protein can be found at Protein of the Month: alpha-Amylase [PubMed](#).

QuickGO

Function

cation binding (GO:0043169)
catalytic activity (GO:0003824)

Process

carbohydrate metabolism (GO:0005975)

Alignment

Domain organisation

Seed (54) Full (3295)

Coloured alignment



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Species Distribution

[View alignments & domain organisation by species](#)

Tree depth : Show all levels



The trees were generated using Quicktree
To find out more about ATv phylogenetic tree-viewer [click here](#)

Database References

[1m7x A; 265; 338;](#)

PDB
You can find out how to set up Rasmol [here](#)

| | | | | |
|-----------------|--|--|--|--|
| | | | | |
| PFAMA | PRC only:Glyco hydro 70 | PRC + PRODOM:PB000186 PB000968 PB004778 PB011311 | | |
| PFAMB | PRC + PRODOM:PB002845 PB008820 PB009903 PB010265 | PRC only:PB001204 PB001218 PB001471 PB001764 PB001802 PB003126 PB007739 PB014225 PB015923 | | |
| | | PRODOM only:PB017201 PB020058 PB026132 PB034852 PB040243 PB052316 PB065724 PB070713 PB087946 PB090253 PB093391 | | |
| | | PB127233 PB131863 PB145968 PB152231 PB162562 PB178513 PB180054 PB180379 PB181121 PB184132 PB184884 | | |
| | | PB185897 | | |
| PRINTS | PR00110 | | | |
| CAZY | GH 13 | | | |
| HOMSTRAD | ghf13 | | | |
| SYSTEMS | Alpha-amylase | | | |
| PANTHER | Alpha-amylase | | | |

| Pfam specific information | |
|---------------------------|-----------------------------------|
| Author of entry | Somhammer ELL, Griffiths-Jones SR |
| Type definition | Domain |
| Source of seed members | Swissprot |
| Average Length | 22 |
| Average %id | 43.19 |
| Average Coverage | CHANGE% |
| HMMER build information | |
| Pfam_ls [Download HMM] | Pfam_fs [Download HMM] |
| Gathering cutoff | -92.6 -92.6 |
| Trusted cutoff | -92.6 -92.6 |
| Noise cutoff | -92.9 -92.9 |
| Build method of HMM | hmmbuild -F HMM_ls SEED |
| | hmmbuild -f F HMM_fs SEED |

hmmcalibrate --cpu 1 --seed 0 HMM_ls hmmcalibrate --cpu 1 --seed 0 HMM_fs

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Annex 3

Pfam motive of amino acid sequence as disclosed in US 10/573,999

Annex 3



Pfam

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Keyword
domain
domain
domain
DNA sequence
Taxonomy
Plant and
Genomes
Cialis
Interaction
Hyperlink directly to the FTP site
View FTP site
Pfam Home page
Browse Pfam
Mail help page
Getting started
Guide to domain
Create your own domain images
Changes from last release
Pfam MySQL documentation
Planned changes to Pfam
FAQ on Pfam
Linking to Pfam
Using Bayevo to view alignments

Walling, 1st, O'Brien
Hib of scales or Ham

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Alphabetic and Numeric Codes

residues]

Trusted matches - domains scoring higher than the gathering threshold (A)

| Domain | Start | End | Bits | Evalue | Alignment | Mode |
|-----------------|-------|-----|--------|---------|-----------|------|
| Isoamylase_N | 125 | 212 | 6.70 | 0.021 | Align | Is |
| Alpha-amylase | 414 | 803 | -76.10 | 0.0025 | Align | Is |
| Alpha-amylase_C | 804 | 897 | 85.10 | 1.9e-22 | Align | Is |

Matches to Pfam-B

| Domain | Start | End | Alignment |
|---------------|-------|-----|-----------|
| Pfam-B 128458 | 1 | 336 | Align |
| Pfam-B 150229 | 337 | 382 | Align |
| Pfam-B 65794 | 412 | 447 | Align |
| Pfam-B 186 | 503 | 640 | Align |

Potential matches - Domains with Evalues above the cutoff

| Domain | Start | End | Bits | Evalue | Alignment | Mode |
|--------------|-------|-----|------|--------|-----------|------|
| Isoamylase_N | 338 | 364 | 0.60 | 7.1 | Alian | fs |
| DUE1247 | 576 | 589 | 2.40 | 0.9 | Alian | fs |

Alignments of Pfam-A domains to HMMs

Jalview Java alignment view at

[http://www.ebi.ac.uk/Tools/jalview/jalview.html](#)

Format for fetching alignments to seed

► Alignment of Ispamylase_N vs UNKNOWN-QUERY/125-212

```

*->plGAhydpddtvdGevggnFrWAPnAerVsLw1dfnnngkdwdge
  +G  h+  ++
  v  F  +WAP+A+  +L+dFn+  w  ++
  UNKNOWN-QU  125  LMGMHRNIOH-----RVDFILEWAPGARYCALITGDFNG----WSTT 160

  ehpmkrs..etnrdgGvWell1pgdnGpd1khgsGgvrykYrittgpdape
  +  +++  +  ++d G+W + 1++  1 +g  +  +
  UNKNOWN-QU  161  GNCAREGhfG-HDDGYWFILED----KLREGEPPDKLYFQQYNYA---  202

  eGhrfneiikl1DPYA<-*
  e  ++k D  +
  UNKNOWN-QU  203  -----EDYDKGDTGI  212

```

► Alignment of Alpha-amylase vs UNKNOWN-QUERY/414-803

```

*->qiypdrFewGpsndpsssyGsaagcdrvP tqielerkyEGGD1qG
  +  +  F+
  UNKNOWN-QU  414  EPKVSSEN-----DF 423

  iidkldiyIkdlGvtaiW1sPifeniaptaspqkdstsyyhgYdttDykkin
  i  +  l+  k++G++a  ++  +e  +  +  qY  +t+++++
  UNKNOWN-QU  424  ISKVLEHVKEAGYNATQ1IGVVE----HKD---YFTVGYRVTNFYAV-  463

  QGvdpryGtkadfksLidaaharGIKvi1DvVpNHTsdehawedvtfques
  +ryGt  +dfk+L+d+ah +G  v 1 +v  H+  ++  a  e+v  +
  UNKNOWN-QU  464  ---SSRYGTPDDFKRLVDEAHLGLLVLFILEIV--HS-YAAADEMV---G  503

  nkskdnpedygdyiieWfpqk1snpgnpyssptnwqshkfdiyfdqsnwsd
  +  d  d+y+  ++gk+  +++  +  +  +
  UNKNOWN-QU  504  LSLFDGA---NDCYF--HTGKR----GHHK-----FWGTRMEKY  533

  gdengdeyylh1flvgLpDlntNepe.....Vr

```


| | | |
|------------|---|---|
| UNKNOWN-QU | g1PqaGqqktyrdvlginsDaeeyGGsg.1gnsgctGrkptvvtVtceep g++t+ag +y++ 1 n+D+ +yGG g 1g+++ t r+ +++ 837 GVEEAG--EYQVTL--NTDENKYGGRLGHDQNTQRT---ISR---R 874 | wydGrkpySwgltl1pplsalvLhlkak<-* dG +++ 1++ 1P++sa v++1 + 875 A-DGM-RFC--LEVPLPSRSQAQVYKL--T 897 |
| UNKNOWN-QU | | |

► Alignment of Isoamylase N vs UNKNOWN-QUERY/338-384

Alignment of DUF1247 vs UNKNOWN-QUERY/576-589

| | | | |
|------------|-----|--|-----|
| UNKNOWN-OU | 576 | *->FrknrkthrDeyve<-* F++++++VV+ FTGMDNEYCNQYVD | 589 |
|------------|-----|--|-----|

an exit linked to swiss of arm

Format for fetching alignments to β family

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Query 1-336 matching **fram-B 128458**

| temp | 1 | MLSLSDSIRISSPLSDRSLSFLSQTGSRTSRLQKFVRSSRRARVSRCRCSA | 50 | |
|-------|---|---|----|--|
| | | MLSLSDSIRISSPLSDRSLSFLSQTGSRTSRLQKFVRSSRRARVSRCRCSA | | |
| Query | 1 | MLSLSDSIRISSPLSDRSLSFLSQTGSRTSRLQKFVRSSRRARVSRCRCSA | 50 | |

| | | | |
|-------|-----|---|-----|
| temp | 51 | TEQPPPQRKRQRPEKYKQSEEKIDPVGFLSKYGITKAFQAFLRERYK | 100 |
| Query | 51 | TEQPPPQRKRQRPEKYKQSEEKIDPVGFLSKYGITKAFQAFLRERYK | 100 |
| temp | 101 | SLKDLKDEILTRHFSLKEMSTGYELMGMRN1QHRVDLEWAPGARYCAL | 150 |
| Query | 101 | SLKDLKDEILTRHFSLKEMSTGYELMGMRN1QHRVDLEWAPGARYCAL | 150 |
| temp | 151 | IGDFNGWSTTGNCAREGHGHDDGYWFILEDKLREGEEDPKLYFQQYN | 200 |
| Query | 151 | IGDFNGWSTTGNCAREGHGHDDGYWFILEDKLREGEEDPKLYFQQYN | 200 |
| temp | 201 | YAEDYDKGDTGIVVEIFKKKANDEYWEPGEDRFIKSRYEVAKLYEEMFG | 250 |
| Query | 201 | YAEDYDKGDTGIVVEIFKKKANDEYWEPGEDRFIKSRYEVAKLYEEMFG | 250 |
| temp | 251 | PNGPQTEEELAMPDAATTRYKTWKEQQQKKDPASNLPSPYDVSKEYDIY | 300 |
| Query | 251 | PNGPQTEEELAMPDAATTRYKTWKEQQQKKDPASNLPSPYDVSKEYDIY | 300 |
| temp | 301 | NIIGDPESFKFRMKQPP1AYWLETKGRKGMLQKY | 336 |
| Query | 301 | NIIGDPESFKFRMKQPP1AYWLETKGRKGMLQKY | 336 |

►Query Query/412-447 matching Pfam-B_150229

| | | | |
|-------|-----|---|-----|
| temp | 337 | MPALPHGSKXKRVYFNTPNGPLERPAWANFV1PDADGMALAVHWE | 392 |
| Query | 337 | MPALPHGSKXKRVYFNTPNGPLERPAWANFV1PDADGMALAVHWE | 392 |

►Query Query/412-447 matching Pfam-B_65794

| | | | |
|-------|-----|-------------------------------------|-----|
| temp | 412 | GQEPKVSSFNDFTSKVLPHVKEAGYNATQ1IGVEH | 447 |
| Query | 412 | GQEPKVSSFNDFTSKVLPHVKEAGYNATQ1IGVEH | 447 |

►Query Query/503-640 matching Pfam-B_186

temp 503 GLSLFDGANDCYFHTGKRHHKFWGTRMFKYGDPDVLFHFLLSNLNNWVEE 552
GLSLFDGANDCYFHTGKRHHKFWGTRMFKYGDPDVLFHFLLSNLNNWVEE
Query 503 GLSLFDGANDCYFHTGKRHHKFWGTRMFKYGDPDVLFHFLLSNLNNWVEE 552

temp 553 YHVDGFFHFSLSMMLYTHNGFASFTGDMDEYCNQYVDEALLYLILANEV 602
YHVDGFFHFSLSMMLYTHNGFASFTGDMDEYCNQYVDEALLYLILANEV
Query 553 YHVDGFFHFSLSMMLYTHNGFASFTGDMDEYCNQYVDEALLYLILANEV 602

temp 603 LHALHPNVITIAVDAFLYPGLCDPTSQQGLGFDFYFANL 640
LHALHPNVITIAVDAFLYPGLCDPTSQQGLGFDFYFANL
Query 603 LHALHPNVITIAVDAFLYPGLCDPTSQQGLGFDFYFANL 640

Alon@jamb

Comments or questions on the site? Send a mail to pfam-help@sanger.ac.uk

Annex 4



NCBI Nucleotide [Sign In] [R]

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM

Search **Nucleotide** for

Display **GenBank** Show 5 Send to Hide: sequence all but gene, CDS and mR

Range: from to Reverse complemented strand Features: Refresh

1: [A22363](#). Reports Plasmid DNA from ...[gi:904243]

Links

Features Sequence

LOCUS A22363 2909 bp DNA linear PAT 04-MAY-1995
 DEFINITION Plasmid DNA from patent WO9214827.
 ACCESSION A22363
 VERSION A22363.1 GI:904243
 KEYWORDS
 SOURCE synthetic construct
 ORGANISM synthetic construct
 other sequences; artificial sequences.
 REFERENCE 1 (bases 1 to 2909)
 AUTHORS
 TITLE PLASMIDS CONTAINING DNA-SEQUENCES THAT CAUSE CHANGES IN THE CARBOHYDRATE CONCENTRATION AND THE CARBOHYDRATE COMPOSITION IN PLANTS, AS WELL AS PLANT CELLS AND PLANTS CONTAINING THESE PLASMIDS
 JOURNAL Patent: WO 9214827-A 1 03-SEP-1992;
 FEATURES Location/Qualifiers
 source 1..2909
 /organism="synthetic construct"
 /mol_type="unassigned DNA"
 /db_xref="taxon:32630"

ORIGIN

```

1 tcaggagcgg tcttggata ttcttccac cccaaaatca agagttagaa aagatgaaag
61 gatgaagcac agttcagcta ttccgctgt ttgaccgat gacaattcga caatggcacc
121 cctagaggaa gatgtcaaca ctgaaaatat tggccctcta aatttggatc caactttgga
181 accttatcta gatcactca gacacagaat gaagagatg gtggatcaga aaatgctcat
241 taaaaatgatggggccccc ttgaggaatt tgctcaaggt tattttaaat ttggattcaa
301 cagggaaat ggttgcata gttatcgta atgggctct gctgctcagg aagcagaagt
361 tattggcgt ttcaatggta ggaacggttc taaccacatg atggagaagg accagttgg
421 tggttggagt attagaattc ctgatgttga cagtaagccca gtcattccac acaactccag
481 agttaagttt cgtttcaaacc atggtaatgg agtgtggta gatcgatcc ctgcttggat
541 aaagtatgcc actgcagacg ccacaaagt tgcagcacca tatgtatgtg tctactggaa
601 cccaccaccc tcagaaaggt accacttcaa ataccctcgcc cctccaaac cccgagccccc
661 acgaatctac gaagcacatg tcggcatgag cagctctgag ccacgtgtaa attcgatcg
721 tgagtttgcgat gatgtatggtt tacctcgat taaggcaat aactataata ctgtccagtt
781 gatggccata atggAACATT cttaactatgg atcatttggat tatcatgtt caaacttttt
841 tgctgtgagc aatagatatg gaaacccggaa ggacctaaag tatctgtatgataaagcaca
901 tagcttgggt ttacagttc tggtggatgt agttcacatg catgcacca ataatgtcac
961 tgatggccctc aatggcttgcgat tttttggcoa aggttctcaa gaatcctact ttcatgtctgg
1021 agagcgaggg taccataatg tggtggatgt caggctgttc aactatggca atttggaggt
1081 tcttcgttccatc cttcttccaa acttggatgt gtggctagaa gagtataact ttgacggatt
1141 tcgatgttgcgat ggaataactt ctatgtgtat tggttcatcat ggaatcaata tggatttac
1201 agggaaactat aatggatatt tcagcgaggg tacagatgtt gatgtgtgg tctatttat
1261 gttggccat aatctgattt acaagatttt cccagacgca actgttatttgg cggaaatgtt
1321 ttctgttgcgat ccggccctta gcccggctgt ttctggatgg ggaattgtt ttgattaccg
1381 cctggcaatg gcaatcccag ataagtggat agattattt aagaataaga atgtgaaga
1441 ttggccatg aaggaagttt catcgatgtt gacaaatagg agatataacg agaagtgtat
1501 agcatatgcg gagagccatg atcagtttatg tgctggatgc aagaccatttgc catttctct
1561 aatgaacaaa gagatgtattt ctggcatgttc ttgtttgaca gatgttttc ctgttggat
1621 tgcaggaattt ggcgttgcgat agatgttcaatggcccttgg gggaggagg
1681 ggtacccatc aaatggatgttgc ggcattccatgatgc ttccctgttgc

```

| | | | | | | |
|------|-------------|-------------|-------------|--------------|-------------|-------------|
| 1741 | agggcaataa | ttggagttat | gacaaaatgta | gacgccagtg | gaacctcgca | gatagcgaac |
| 1801 | actttagata | caagtttatg | aatgcatttg | atagagctat | gaattcgctc | gatgaaaagt |
| 1861 | tctcattcct | cgcatcagga | aaacagatag | taagcagcat | ggatgtatgt | aataagggtt |
| 1921 | ttgtgtttga | acgtggtgac | ctggtatttg | tattcaactt | ccacccaaat | aacacatacg |
| 1981 | aagggtataa | agttggatgt | gacttgccag | ggaagtacag | agttgcactg | gacagtgtatg |
| 2041 | cttgggaatt | tggtgccat | ggaagagctg | gtcatgatgt | tgaccatttc | acatcaccag |
| 2101 | aaggaataacc | tggagttcca | gaaacaaaatt | tcaatggtcg | tccaaaattcc | ttcaaaagtgc |
| 2161 | tgtctcctgc | gcgaacatgt | gtggcttatt | acagagttga | tgaacgcatg | tcataaactg |
| 2221 | aagattacca | gacagacatt | tgttagtgagc | tactaccaac | agccaatatac | gaggaaagtg |
| 2281 | acgagaaaact | taaagattca | tcatctacaa | atatcagtagc | atcatctaca | aaaaatgtt |
| 2341 | attacagagt | tgtgaacgc | atgtcagaag | ctgaagatta | ccagacagac | atttgttagtg |
| 2401 | agctactact | accaacagcc | aatatcgagg | agagtgcacga | gaaacttgtat | gattcattat |
| 2461 | ctacaatata | cagtaacatt | ggtcagactg | ttgttagttc | tgttgaggag | agagacaagg |
| 2521 | aacttaaaga | ttcaccatct | gtaaagcatca | ttagtgtatgc | tgttccagct | gaatgggctg |
| 2581 | attcgatctgc | aaacgtctgg | ggtgaggact | agtcaagatga | ttgatcgatc | cttctacgtt |
| 2641 | ggtgatctcg | gtccgtgcatt | gatgtcttca | gggtggtagc | attgactgtat | tgcatacatag |
| 2701 | tttttttttt | tttttttaag | tatttcctct | atgcataatata | ttagcatcca | ataaaatttac |
| 2761 | ttgttgttgt | acatagaaaa | agtgcatttg | catgtatgt | tttctctgaa | attttccccca |
| 2821 | gttttgggtgc | tttgcctttg | gagccaagtc | tctatatgt | ataagaaaac | taagaacaaat |
| 2881 | cacatataata | aaatgttagt | agattacca | | | |

11

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Last update: Thu, 26 Jun 2008 Rev. 132340

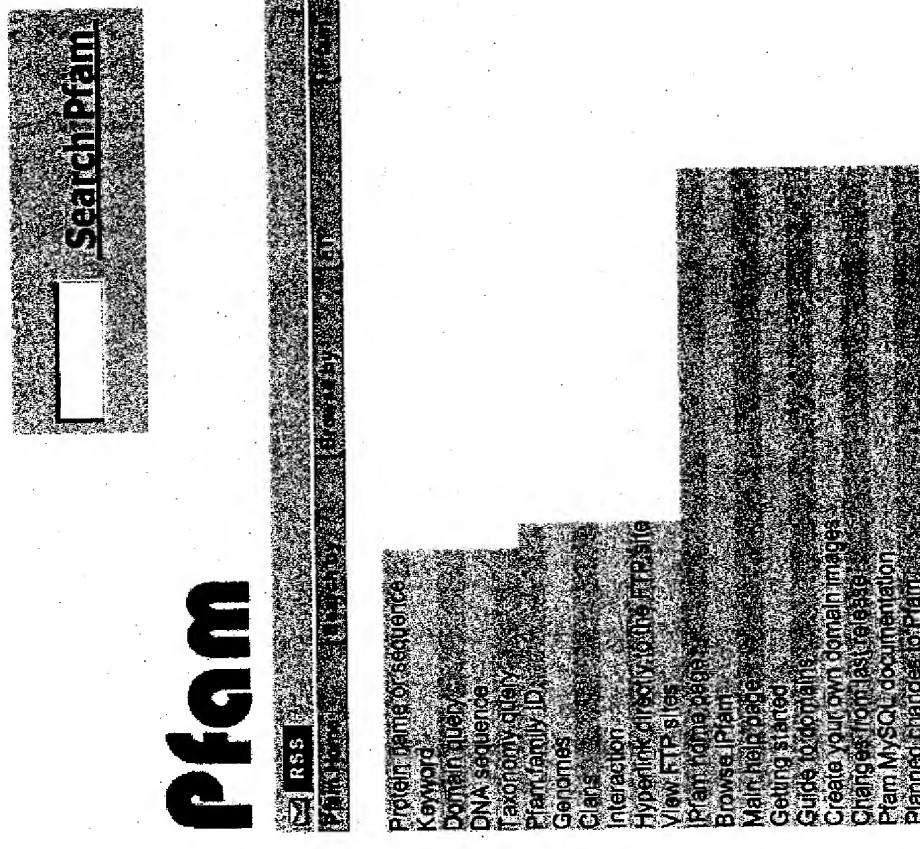
Annex 5



Annex 5

Pfam motive of amino acid sequence deduced from the nucleotide sequence as disclosed in

EP 1103617 A2.doc



AC on PAMI
Ability of PAMI to
serve as a model for
other countries

Alphamethylase 546

Residues]

Trusted matches - domains scoring higher than the gathering threshold (A)

| Domain | Start | End | Bits | Evalue | Alignment | Mode |
|---------------|-------|-----|--------|---------|-----------|------|
| Isamylase_N | 76 | 160 | 95.90 | 1.1e-25 | Allan | Is |
| Alpha-amylase | 201 | 532 | -47.20 | 0.00015 | Allan | Is |

Matches to Pfam-B

| Domain | Start | End | Alignment |
|--------------|-------|-----|--------------|
| Pfam-B_1450 | 21 | 68 | <u>Align</u> |
| Pfam-B_1269 | 186 | 210 | Align |
| Pfam-B_65784 | 211 | 251 | <u>Align</u> |
| Pfam-B_186 | 295 | 504 | <u>Align</u> |
| Pfam-B_1204 | 505 | 544 | <u>Align</u> |

Potential matches - Domains with **Eval values above the cutoff**

| Domain | Start | End | Bits | Evalue | Alignment | Mode |
|--------|-------|-----|------|--------|-----------|------|
| PRF | 55 | 67 | 1.70 | 0.67 | Align | fs |

| IGPS | 268 | 288 | 3.60 | 0.73 | Align | fs |
|------|-----|-----|------|------|-------|----|
|------|-----|-----|------|------|-------|----|

Alignments of Pfam-A domains to HMMs

Java view Java alignment view er

Format for fetching alignments to seed

► Alignment of Isoamylase_N vs UNKNOWN-QUERY/76-160

```
*->plGAhydpddtvdGevgvnFrwApnAervsLvldfnnngqkdwdge
    ++G+++          e+g ++r+MAF+A+++++dfnt+          ++g
UNKNOWN-QU 76 KFGNR-----EDGCIVREWAEEAQEEAEVIGDFNG---RNGS 110

ehpmkrsetnrdggGwElfIpgd.ng.pdlkhgsGgvrYkYritgpdape
    h m+          ++ +GvW++ +p d + +p +th+s          r+k+r++++
UNKNOWN-QU 111 NHMME---KDQFGVWSIRIP-DvDSkPVIPHNS---RVKFRFKHGN--- 149

eGhrfneiklklDPYA<-*+
    G ++++++++
UNKNOWN-QU 150 -G---VWVDRIPAWI 160
```



► Alignment of Alpha-amylase vs UNKNOWN-QUERY/201-532

```
*->qiypdrFewgDpsndps.ssyGsaagcrdrePtkqgielrkfGGD1q
    +s p+ +sy
    iy
UNKNOWN-QU 201 RYEAHVG--MSSSEPRvNSYR-----E 221

Giidk1dyIkdlGvtaiW1sPifenipntaspqkdstsyhgYdttDYKKi
    d 1+ +k ++ +1+ i e          + ++ +qY+t++++
UNKNOWN-QU 222 FADDVLFRIKANNNTVQLMAIME----HSY---YGSFGYHVTNEFAV 262

nQGvdpdryGtkaclfksLidaaharGIKwi1DvVpNHTsdehawedvtfqe
    ryG+ +d+k Lid+ah+G +v++DvV H+ ++ +
UNKNOWN-QU 263 ----SNRGNPEDIKYLIDKAHSLGQLQVLVDVV--HS-HASNN----- 298
```

->DFeLLAKKYqGhL<-
D ++L +KY G+L
DQKMLIEKYEGPL
UNKNOWN-QU 55

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Alignment of PRF vs UNKNOWN-QUERY/55-67

->sdeLLeeLyeyArsJGMevLV<-
+ e L+ L+ A+s1G+vvV
UNKNOWN-QU 268 NPEDLKYLIDKAHSGLQVLV 288

Alignments of Pfam-B domains to best-matching to Pfam-B sequence

Format for fetching alignments to Pfam-B families:

[Hypertext linked to swisspfam](#)

►Query Query/21-68 matching Pfam-B_1450

temp 21 LEEDVNTEIGLNLDPTEPYLDHFRHRMCKRYVDQKMLIEKYEGPLE 68
LEEDVNTEIGLNLDPTEPYLDHFRHRMCKRYVDQKMLIEKYEGPLE
Query 21 LEEDVNTEIGLNLDPTEPYLDHFRHRMCKRYVDQKMLIEKYEGPLE 68



►Query Query/186-210 matching Pfam-B_1269

temp 186 RYHFKYPRPKPRAPRIYEAVGMS 210
RYHFKYPRPKPRAPRIYEAVGMS
Query 186 RYHFKYPRPKPRAPRIYEAVGMS 210



►Query Query/211-251 matching Pfam-B_65794

temp 211 SSEPRVNSYREFADDVLPRIKANNYNTVQLMAIMEHSYYGS 251
SSEPRVNSYREFADDVLPRIKANNYNTVQLMAIMEHSYYGS
Query 211 SSEPRVNSYREFADDVLPRIKANNYNTVQLMAIMEHSYYGS 251



►Query Query/295-504 matching Pfam-B_186

->sdeLLeeLyeyArsJGMevLV<-
+ e L+ L+ A+s1G+vvV
UNKNOWN-QU 268 NPEDLKYLIDKAHSGLQVLV 288

temp 295 ASNNTDGLNGFDIGQGSQESYFHAGERGYHKLWDSRLENYANWEVLRFL 344
ASNNTDGLNGFDIGQGSQESYFHAGERGYHKLWDSRLENYANWEVLRFL

Query 295 ASNNTDGLNGFDIGQGSQESYFHAGERGYHKLWDSRLENYANWEVLRFL 344

temp 345 LSRLRWLLEEYNFDGFRFDGITSMLYVHKGINMGFTGNMNEYFSEATDVD 394
LSRLRWLLEEYNFDGFRFDGITSMLYVHKGINMGFTGNMNEYFSEATDVD

Query 345 LSRLRWLLEEYNFDGFRFDGITSMLYVHKGINMGFTGNMNEYFSEATDVD 394

temp 395 AVVYMLANNLILHKIFPDATVIAEDVSGMPGLSRPVSEGGIGFDYRLAMA 444
AVVYMLANNLILHKIFPDATVIAEDVSGMPGLSRPVSEGGIGFDYRLAMA

Query 395 AVVYMLANNLILHKIFPDATVIAEDVSGMPGLSRPVSEGGIGFDYRLAMA 444

temp 445 IPDKWIDYLKNNKNDEDWSMKEVTSSSLNRRYTEKCIAYAESHDQSIVGDK 494
IPDKWIDYLKNNKNDEDWSMKEVTSSSLNRRYTEKCIAYAESHDQSIVGDK

Query 445 IPDKWIDYLKNNKNDEDWSMKEVTSSSLNRRYTEKCIAYAESHDQSIVGDK 494

temp 495 TIAFLLMNKE 504
TIAFLLMNKE
Query 495 TIAFLLMNKE 504

[REDACTED]

►Query Query//505-544 matching Plan-B 1204

temp 505 MYSGMSCLTDASPVVDAGIALDKMIFFFHNGLRRGVPOF 544
MYSGMSCLTDASPVVDAGIALDKMIFFFHNGLRRGVPOF

Query 505 MYSGMSCLTDASPVVDAGIALDKMIFFFHNGLRRGVPOF 544

[REDACTED]